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### **Collagen $\alpha$ -Tocopherulate for Topical Applications: Preparation, Characterization,**

Questa è la Versione finale referata (Post print/Accepted manuscript) della seguente pubblicazione:

*Original Citation:*

Collagen  $\alpha$ -Tocopherulate for Topical Applications: Preparation, Characterization, and Antioxidant Activity Evaluation / S. Trombino; R. Cassano; T. Ferrarelli; B. Isacchi; A.R. Bilia; N. Picci. - In: MACROMOLECULAR RESEARCH. - ISSN 1598-5032. - STAMPA. - (2012), pp. 939-943.

*Availability:*

This version is available at: 2158/778488 since:

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## Collagen $\alpha$ -Tocopherulate for Topical Applications: Preparation, Characterization, and Antioxidant Activity Evaluation

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Received July 26, 2011; Revised December 12, 2011; Accepted December 20, 2011

**Abstract:** The aim of this work was to synthesize collagen  $\alpha$ -tocopherulate for topical treatments. Collagen is a natural polymer largely applied to enhance wound repair, and  $\alpha$ -tocopherol is a known antioxidant used for the treatment of various skin diseases. Linking the two through covalent bonds could improve  $\alpha$ -tocopherol transport and metabolic stability, reduce the rate of degradation, and ensure a longer persistence than free antioxidant. The new biopolymer was prepared by a solid-phase synthesis employing a carboxypolystyrene resin. Its antioxidant activity in rat liver microsomal membranes was also evaluated. The obtained collagen-based biomaterial represents a new prodrug of  $\alpha$ -tocopherol that could be used for various biomedical applications.

**Keywords:** solid phase synthesis,  $\alpha$ -tocopherol protein conjugate, antioxidant activity.

### Introduction

Over the last decades, significant progresses have been made in the development of biodegradable polymeric materials for biomedical applications. They are, at present, the structures of choice for the development of therapeutic devices, such as temporary prostheses, porous three-dimensional structures, vehicles for the controlled and/or delayed release of drugs and materials for tissue engineering.<sup>1</sup> Actually, there are many biocompatible natural or synthetic materials that can undergo hydrolytic or enzymatic degradation for biomedical applications.<sup>2</sup> Among these, collagen is considered as one of the most useful biomaterials. Its excellent biocompatibility and its safety, mainly due to the biodegradability and weak antigenicity,<sup>3</sup> make it one of the primary resources in biomedical applications. The use of collagen as a drug delivery system is very comprehensive and diverse: the main applications are collagen shields in ophthalmology, sponges for burns/wounds, mini-pellets, nanoparticles and tablets for protein, and for transdermal delivery. In particular collagen sponges have been very useful in the treatment of severe burns and as a dressing for many types of wounds, such as pressure sores, donor sites, leg and decubitus ulcers as well as for *in vitro* test systems.<sup>4</sup> Collagen plays an important role in the formation of tissues and organs, and is involved in various functional expressions of cells. It is a primary structural constituent of vertebrates, as well as the

most abundant protein in mammals, accounting for roughly 20%-30% of the total protein in the body. It is present in tissues such as skin, bones, tendons and ligaments, with a predominantly mechanical function, and preserves, in addition, the structural integrity of all internal organs. The structural unit of collagen is represented by the tropocollagen, a protein with a molecular mass of approximately 285 kDa, composed of three polypeptide chains that are associated with left-handed pattern to form a right-handed triple helix. The largest amount of collagen, which is currently used for biomedical applications, are obtained from the skin of cattle or swine or bovine Achilles tendon. One disadvantage of these collagen-based biomaterials and the limiting factor for their clinical applications is their mild immunogenicity, given by the composition of the terminal region of the chains. The immune response was found to be different depending on the species from which the collagen has been isolated, or on the implant site; the immunogenicity was reduced by pretreatment of the protein before application. Other limits to its use can also result from the high cost of pure collagen, its poor chemical stability, the risk of transmission of infectious diseases due to allogeneic or xenogeneic origin of the material. Several recombinant systems are currently being developed for the production of human collagen.<sup>5</sup> This last undergoes enzymatic degradation within the body by enzymes such as collagenase and metalloproteinase, releasing the corresponding aminoacids that compose itself. Since collagen is the main component of the extracellular matrix and acts as a natural substrate for cells connection, prolifer-

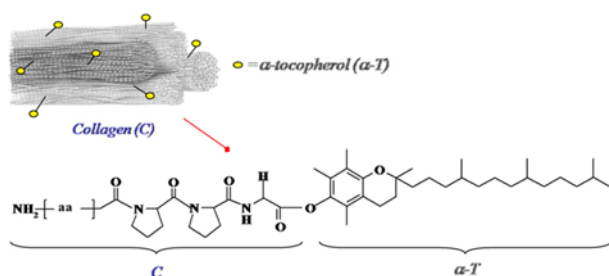
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ation and differentiation, it is understandable the increasing interest in using it as an ideal material for the medical tissutal engineering. When tissues are injured due to trauma, collagen is needed to repair the defect and restore the structure and hence function. An excessive deposition of collagen can induce fibrosis, compromising the structure and the function of the damaged site. Conversely, if the quantity of deposited collagen is insufficient, the tissue hardly undergoes complete regeneration. A spongy matrix, containing collagen and oxidized cellulose, has been recently introduced in the European market for the treatment of exuding wounds in patients with diabetic ulcers.<sup>6</sup> Of great importance also its use as a substitute for skin in the treatment of burn injury.<sup>7</sup> Wound healing is a highly specified process that starts with the formation of granulation tissue and ends with scar formation. In most of the cases, the complication in wound healing is the inflammation that results in a continuous generation of reactive species, such as the superoxide radical ( $O_2^-$ ) or the non-radical hydrogen peroxide ( $H_2O_2$ ), which are now strongly implicated in the pathogenesis of chronic wounds.<sup>8</sup> Such a property is of significance to the wound healing process, as despite the beneficial role that ROS (reactive oxygen species) play in killing invading microbial pathogens, excessive overproduction of ROS can be detrimental to the host tissues by inactivating enzymic antioxidants and significantly depleting non enzymic antioxidants levels.<sup>9</sup> Evidence for the potential role of oxidants in the pathogenesis of many diseases suggests that antioxidants may be of therapeutic use in these conditions.

The aim of the present work was to obtain a useful biomaterial antioxidant as a carrier system through the functionalization of collagen with  $\alpha$ -tocopherol (vitamin E), a known antioxidant molecule (Figure 1). The obtained biopolymer is an  $\alpha$ -tocopherol prodrug that could be used in the preparation of unconventional collagen-based drug delivery systems. Particularly, the synthesis was carried out in solid phase by using a carboxypolystyrene resin.

## Experimental

**Materials.** All solvents of analytical grade, were purchased from Carlo Erba Reagents (Milan, Italy): dichloromethane,



**Figure 1.** Schematic representation of collagen  $\alpha$ -tocopherol-linked.

diethylether, *N*-methylpyrrolidone, thionyl chloride, collagen from bovine Achilles tendon (freeze-dried, insoluble),  $\alpha$ -tocopherol, dicyclohexylcarbodiimide (DCC), trifluoroacetic acid, potassium chloride (KCl), ethylenediaminetetraacetic acid (EDTA), sucrose, 4-2-hydroxyethyl-1-piperazineethanesulfonic acid (HEPES), trichloroacetic acid (TCA), hydrochloric acid, butylated hydroxytoluene (BHT), *tert*-butyl hydroperoxide (*tert*-BOOH), and 2-thiobarbituric acid (TBA) were purchased from Sigma-Aldrich (Sigma Chemical Co, St. Louis, MO, USA). Resin (carboxypolystyrene HL, 100-200 MESH, 1% DVB) was purchased from Merck AG.

Infrared spectra were recorded on KBr pellets using a FT-IR spectrometer Perkin-Elmer 1720. UV-vis spectra were realized through a UV-530 JASCO spectrophotometer. The samples were lyophilized utilizing a "Freezing-drying" Micro moduly apparatus, Edwards. The calorimetric analyses (DSC) were performed using a Netzsch DSC200 PC.

**Collagen Derivatization.** The derivatization reaction<sup>10,11</sup> was carried out in a vial Disa, suitably flamed, under stirring and nitrogenum atmosphere at room temperature for 3 h. In order to activate the carboxylic function available on the resin, we generated the corresponding acyl chloride by adding thionyl chloride ( $SOCl_2$ , 0.022 mL, 0.3 mmol) in slight excess in a ratio of 1.5:1 compared to the resin (0.2 mmol; 0.2 g). As reaction solvent we used *N*-methylpyrrolidone, the only one that was able to solubilize the collagen under heat treatment (70 °C) and stirring for a minimum of 40 min. After the activation of carboxylic groups present on the resin with thionyl chloride, collagen (0.008 g) was added. In this way the protein was covalently linked to the resin; afterthat  $\alpha$ -tocopherol and a coupling agent such as dicyclohexylcarbodiimide (DCC) were added. We used a slight excess of antioxidant (0.3 mmol, 0.129 g) respect to the resin (0.2 mmol) and DCC (0.3 mmol, 0.062 g) in equal amounts with  $\alpha$ -tocopherol. Reaction was conducted at room temperature for 72 h. In order to obtain the cleavage of the derivatized protein from the resin, 0.5 mL of trifluoroacetic acid (TFA) 5% in 10 mL of dichloromethane (DCM) were added. This reaction was conducted under nitrogen atmosphere and continuous magnetic stirring. We performed a filtration under reduced pressure on a porous filter. We got a clear and yellow filtrate. The resin, after filtration and washing has been retained for reuse. The obtained product was washed with several aliquots of diethyl ether, extracted with chloroform, dried at reduced pressure and characterized (Yield: 70%).

**Antioxidant Activity Evaluation through Rat Liver Microsomal Membranes.** The antioxidant activity in inhibiting the lipid peroxidation, in rat-liver microsomal membranes, induced *in vitro* by a source of free radicals such as *tert*-butyl hydroperoxide (*tert*-BOOH), was evaluated. Liver microsomes were prepared from Wistar rats by tissue homogenization with 5 volumes of ice-cold 0.25 M sucrose containing 5 mM Hepes, 0.5 mM EDTA, pH 7.5 in a Potter-Elvehjem

homogenizer.<sup>12</sup> The microsomal membranes were isolated by the removal of the nuclear fraction at 8,000 g for 10 min, and by the removal of the mitochondrial fraction at 18,000 g for 10 min. The microsomal fraction was sedimented at 105,000 g for 60 min, and the fraction was washed once in 0.15 M KCl, and was collected again at 105,000 g for 30 min.<sup>13</sup> The membranes, suspended in 0.1 M potassium phosphate buffer, pH 7.5, were stored at -80 °C. Microsomal proteins were determined by the Bio-Rad method.<sup>14</sup>

Aliquots of collagen derivative and of  $\alpha$ -tocopherol in the range of 0.5–6 mg/mL were added to the microsomes. These microsomes were gently suspended by a Dounce homogenizer, and then the suspensions were incubated at 37 °C in a shaking bath under air in the dark.

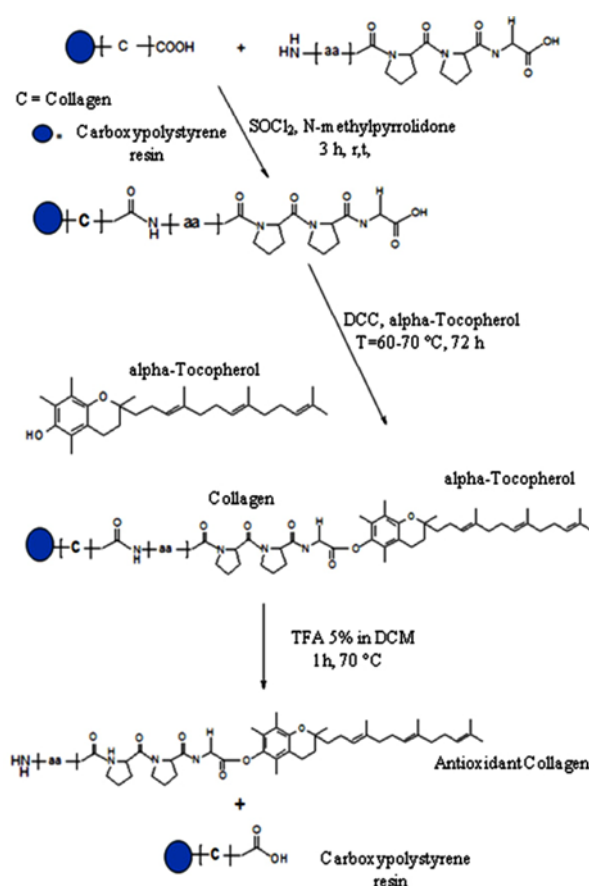
Malondialdehyde (MDA) was extracted and analyzed as indicated.<sup>15</sup> Briefly, aliquots of 1 mL of microsomal suspension (0.5 mg proteins) were mixed with 3 mL of 0.5% TCA and 0.5 mL of TBA solution (two parts 0.4% TBA in 0.2 M HCl and one part distilled water), and 0.07 mL of 0.2% BHT in 95% ethanol. The samples were then incubated in a 90 °C bath for 45 min. After incubation, the TBA-MDA complex was extracted with 3 mL of isobutyl alcohol. The absorbances of the extracts were measured by the use of UV spectrophotometry at 535 nm, and the results were expressed as percent of MDA inhibition, using an extinction coefficient of  $1.56 \times 10^5 \text{ L mmol}^{-1} \text{ cm}^{-1}$ .

## Results and Discussion

As evidenced by the abundance of data in literature, a growing interest is now attributed to the design and implementation of biocompatible systems for pharmaceutical applications. For these purposes, recently more and more attention is directed to the use of natural polymers or compounds synthesized from materials of natural origin that best meet the needs of biocompatibility.<sup>16–18</sup> The purpose of this work was to synthesize a prodrug collagen-based and therefore compatible with tissues to exploit its intrinsic properties in order to convey the active ingredient bound to collagen and facilitate tissue repair.

**Solid Phase Synthesis of Collagen Derivative.** Since the collagen is a highly composite protein, consisting of a huge number of aminoacids, before to functionalize it with an antioxidant, we performed a preventive treatment in order to anchor the protein to a resin acting as a protecting group of collagen terminal amino-group. After the collagen binding to the resin through its amino-terminal group, and activation of its C-terminal group with thionyl chloride, we proceeded to the functionalization of the protein with a known antioxidant agent,  $\alpha$ -tocopherol, through the formation of an ester bond. After that we cleaved the functionalized collagen from the resin (Scheme I).

In particular, we carried out a heterogeneous synthesis: the ester-linkage between resin and protein has been facili-



Scheme I. Synthetic route.

tated by a prior chlorination of -COOH groups of the resin. The formation of acylic chloride, with markedly electrophilic character and hence more reactive than the corresponding carboxyl function was carried out by treatment of the resin, dissolved in *N*-methylpyrrolidone, with thionyl chloride. The choice to anchor the collagen on the resin surface for its functionalization with  $\alpha$ -tocopherol has been considered mainly in order to avoid the difficulties in conducting the synthesis, which could result from the excessive size of the collagen itself. These problems inherent in the protein matrix characteristic would have been difficult to be managed in a more common synthesis in homogeneous phase. Moreover, we chose the heterogeneous synthesis to facilitate the management of several functional groups in the aminoacids side chain that constitute the collagen. Once the N-terminal function of the polypeptide was locked, our attention has been paid to the free functional groups on the other side of collagen itself. Carboxylic acid functions, useful for the functionalization with the  $\alpha$ -tocopherol phenolic hydroxyl group, are not only those C-terminals, but also those exposed in the side chain of the amino acids constituting the protein. The bond between the resin acyl chloride and the  $\alpha$ -tocopherol hydroxyl group is a relationship of ester. It is generated by reaction between the nucleophilic

group (-OH), present on the antioxidant phenolic ring, and the resin -COCl groups with a strong electrophile character. The covalent bond of the antioxidant moiety on the collagen matrix has something very important from the point of view of the use of this product as a potential prodrug. The protein, in fact, possessing itself a therapeutic action, is enriched and strengthened of the antioxidant properties. The resin can be removed from the derivatized collagen by breaking the ester bond. For this purpose we used, a mild acid treatment which do not affect the ester bond between the protein and the antioxidant. The resin was removed by filtration. The filtrate was washed with diethyl ether to induce precipitation of the product and thus allowing its separation and recovery. The spectrum of non-derivatized collagen shows a high amount of functional groups, relating to the numerous side chains of amino acids that make up the structure. However, comparing starting collagen and functionalized one, we noted the presence of typical bands of ester bonds (Figure 2).

The calorimetric analysis for commercial collagen revealed the presence of a large endothermic at 207.1 °C. On the other hand, the DSC spectrum of derivatized collagen, shows the absence of the transition of commercial vitamin E (113 °C) and the presence of a broad peak similar to that observed for the non-derivatized collagen.

**Antioxidant Activity Evaluation.** The collagen derivative was then tested for its antioxidant activity. The results showed that collagen esterified with  $\alpha$ -tocopherol possesses protective activity against free radicals. The ability of collagen derivative in inhibiting lipid peroxidation induced by *tert*-BOOH, was examined in rat liver microsomal membranes over 120 min of incubation. In order to draw comparisons with the non-functionalized collagen, even this has been tested for its antioxidant capacity (data not shown). The ability of the synthesized material to inhibit lipid peroxidation is time-dependent, in addition the collagen derivative maintains the antioxidant activity and trend over time of the commercial  $\alpha$ -tocopherol (Figure 3).

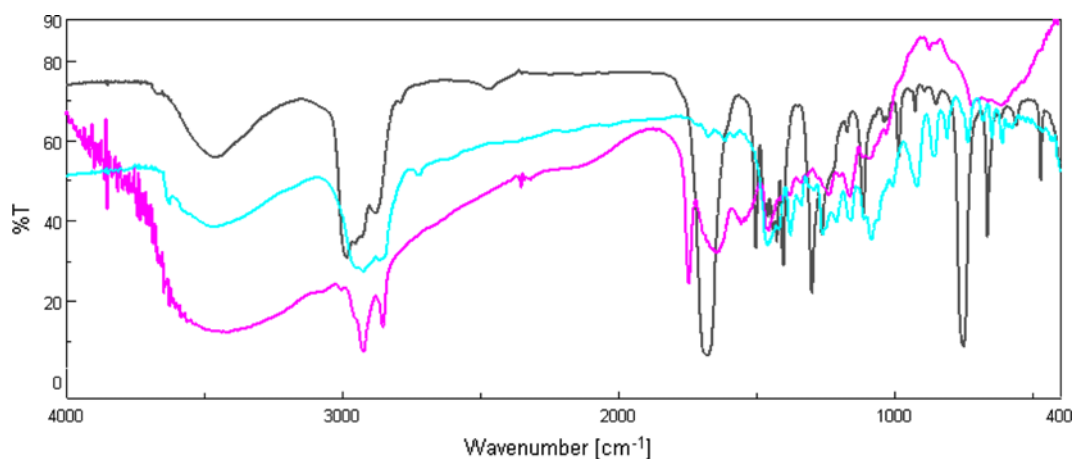


Figure 2. IR analysis of (—) C- $\alpha$ T, (—)  $\alpha$ T, (—) C.

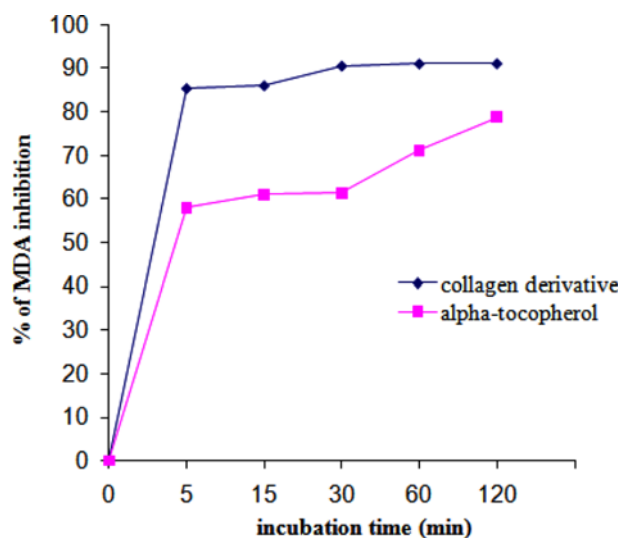


Figure 3. Percentage of inhibition of *tert*-BOOH induced MDA formation in rat microsomal membranes. The microsomal membranes were incubated with  $0.25 \times 10^{-3}$  M *tert*-BOOH at 37 °C under air in the dark. The results represent the mean  $\pm$  standard error of the mean (SEM) of six separate experiments.

## Conclusions

A growing interest is reserved, nowadays, in the research and the development of natural or synthetic materials capable of combining the characteristics of biocompatibility with the site-specificity against target organ.<sup>19-21</sup> For these same reasons we realized a prodrug composed of collagen and  $\alpha$ -tocopherol, in order to obtain a biocompatible carrier with antioxidant properties. Evidence for the potential role of oxidants in the pathogenesis of many diseases suggests that the antioxidants may be of therapeutic use in these conditions. To facilitate the peptide derivatization, we decided to conduct the entire process in heterogeneous phase. On the collagen derivative we carried out some analysis such as FTIR spectroscopy and DSC analysis. Then we tested its antioxi-

dant efficacy on rat liver microsomal membranes, in order to evaluate the antioxidant activity of functionalized collagen. The data obtained by this procedure show that the derivatized product maintains the typical  $\alpha$ -tocopherol antioxidant activity which is able to improve significantly wound healing and protect tissues from oxidative damage.

**Acknowledgment.** This work was financially supported by Academic Funds and MIUR (Programma di Ricerca di Rilevante Interesse Nazionale 2008).

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